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PATENT

Docket No.: 176/60581 (1-11027-845)

Dec. w/attach

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| Applicants | : | Ray et al. | Examiner: J. Zara |
| Serial No. | : | 09/590,968 | Art Unit: 1635 |
| Cnfrm. No. | : | 2086 | |
| Filed | : | June 9, 2000 | |
| For | : | GENE ENCODING SHORT INTEGUMENTS AND USES THEREOF | |

## DECLARATION OF ANIMESH RAY UNDER 37 C.F.R. § 1.132

U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202



Dear Sir:

I, ANIMESH RAY, pursuant to 37 C.F.R. § 1.132, declare:

considered 12/27/02 JZ

1. I received a B.Sc. degree from the Presidency College, Calcutta, India in 1975, an M.Sc. degree in 1977 and an M.Phil. degree in 1979 from Jawaharlal Nehru University, New Delhi, India. I received a Ph.D. degree from Monash University in Melbourne, Australia, in 1985.

2. I have been employed at the University of Rochester, Rochester, New York, as an Assistant Professor in the Department of Biology from 1991-1997, as an Associate Professor from 1997-2001, and as a Visiting Associate Professor from 2001 to the present.

3. I am currently an Associate Professor of Systems Biology at Keck Graduate Institute, Claremont, CA.

4. I am a named inventor of the above patent application.

5. This declaration is submitted to 1) demonstrate that the disclosure of the present application teaches the distinguishing attributes which are shared by the members of the genus comprising nucleic acid molecules encoding a short integuments ("SIN1") protein; and 2) to demonstrate that the *SIN1* nucleic acid molecule of the present application can be used to successfully transform plants,

thereby generating transgenic plants expressing a SIN1 protein, which results in early flowering and the concomitant modulation of fertility and fecundity in such transgenic plants.

**Structural Basis of SIN1 Function**

6. In *Arabidopsis thaliana,* only two genes have been identified whose activities are required in the maternal sporophyte (or female somatic cells) for normal pattern formation during embryo development. One gene identified in this class is the wild-type allele of *SHORT INTEGUMENTS1 ("SIN1").* I previously demonstrated that maternal activity of the *SIN1* gene of *Arabidopsis thaliana* is essential for embryo pattern formation and viability, and that its post-embryonic activity is required for several processes in reproductive development, including flowering time control and ovule morphogenesis (see Ray et al., "Maternal Effects of the *short integuments1* Mutation on Embryo Development," Dev Biol 180: 365-369 (1996), attached herein as Exhibit 1). I have also demonstrated that homozygous *sin1* mutants exhibit defects in flowering time (see Ray et al., "*SHORT INTEGUMENT (SIN1)*, A Gene Required for Ovule Development in *Arabidopsis*, Also Controls Flowering Time," Development 122:2631-2638 (1996), Table I at page 2632 (1996), attached herein as Exhibit 2), delayed transition between developmental states, and failure to coordinate integument formation in ovules (see Figure 1A, attached hereto as Exhibit 3). (Key in Figure 1A: o, outer integuments; i, inner integuments; es, embryo sac.) In wild type ovules, as shown in Figure 1A, the outer integument cell layers entirely cover the inner integument that encloses the embryo sac that contains the egg. Most mutant ovules show uncoordinated growth of both the inner and outer integuments and the nucellus, resulting in the extrusion of the embryo sac, with the egg. These pleiotropic effects suggest that *SIN1* plays a key role in several developmental processes.

7. A 6.2 kilobase pair (kb) cDNA (accession number AF292940) corresponding to the *SIN1* mRNA was isolated from a flower- and seed-enriched cDNA library. The cDNA has a 5,727 bp open reading frame (ORF), a 378 bp 5' untranslated region (UTR), a 74 bp 3' UTR and nine adenines at the 3' end, likely to be from the poly-A tail. The cDNA sequence confirmed the presence of 19 introns and 20 exons. Shown in the lower portion of Figure 1 of the present invention (and Figure 1B of Exhibit 3 herein) is the arrangement of functional motifs of the predicted SIN1 protein: a bipartite N-terminal nuclear localization signal (NLS), a DexH box

RNA helicase C motif, two RNase III catalytic domains, a PIMS (for PIWI Middle domain-SHORT INTEGUMENTS1, PIWI being a family of important plant developmental proteins) domain (identical to the PAZ domain, so called for its occurrence in PIWI, ARGONAUTE, and ZWILLE , all three being developmentally important proteins), and two C-terminal repeats of a dsRNA binding domain. A BLAST search yielded numerous high homology strikes of the above-identified domains of SIN1, shown in Figure 2 of the present application. Each of the three functional domains is strongly conserved within its own family. Structural modeling of the wild-type SIN1 and *sin1* mutant proteins indicates that the RNA helicase domain of SIN1 is particularly important for the protein's function. For example, I have mapped the *sin1-1* and *sin1-2* alleles both to the C-terminal region of the helicase domain, having P415S and I431K substitutions, respectively. Neither amino acid residue had previously been described as crucial for helicase function. Amino acid sequence alignment and homology modeling, using the yeast translation initiation factor 4A (yIF4A) helicase as the template, suggests a similar structural basis for the effects of both point mutations in *sin1:* both amino acid substitutions map on the same face of the predicted helicase domain (see Figure 2, attached hereto as Exhibit 4). The biological function of SIN1 protein, as deduced by the mapping of known mutations (with genetic consequences) into the sequenced and cloned *SIN1* gene, was determined to be essential for plant reproduction and survival, and this demonstration represents the first demonstration of biological function for any member of this class of proteins containing a combination of DexH box helicase domain, PAZ domain, RNase III domain, and dsRNA binding domain.

8. The importance of the RNA helicase domain in SIN1-like development-related plant genes is further supported by the work of Jacobsen et al., "Disruption of an RNA Helicase/RNA III Gene in *Arabidopsis* Causes Unregulated Cell Division in Floral Meristems," Development 126:5231-5243 (1999) (attached hereto as Exhibit 5). Jacobsen discloses a recessive mutant, *carpel factory ("caf"),* which converts the floral meristems of *Arabidopsis thaliana* to an indeterminate state. *Caf* mutants produce extra whorls of stamens, and an indefinite number of carpels (Id. at Abstract). Thus, *CAF* appears to suppress cell division in floral meristems. Furthermore, the *CAF* gene cloned by Jacobsen encodes a protein with similarities to both DexH/DEAD-box type RNA helicases and RNase III proteins, suggesting a mechanism for control of floral meristem proliferation (Id. at 5232, left col., 1st full ¶). The isolated gene of the present invention is identical to the CAF gene (gi6102609)

except for two single base sequencing differences leading to an erroneous designation of one amino acid residue reported in the CAF protein sequence. The correctness of the SIN1 sequence, and its identity to the CAF gene, were confirmed in my laboratory by re-sequencing the *caf* mutant allele of Jacobsen. The identity of *SIN1* to the *SUSPENSOR1 (SUS1)* gene, which is essential for embryogenesis (Schwartz et al., "Disruption of Morphogenesis and Transformation of the Suspensor in Abnormal *suspensor* Mutants of *Arabidopsis,*" Development 120:3235-3245 (at 3243, left col., 1st full ¶ (1994), attached hereto as Exhibit 6) was also shown by PCR analysis and mapping of *sus1* mutations. The presence of the RNA helicase and RNAse III domains suggest that SIN1/SUS1/CAF functions in *Arabidopsis* development through post-transcriptional regulation of specific mRNA molecules. Extrapolating from the known functions of RNA helicase proteins and RNAse III proteins, SIN1/SUS1/CAF1 may function in plant development in mRNA processing (Jacobsen et al., at 5242 last full ¶). Plants may use small dsRNA hairpins (or their cleaved products) as developmental regulators over long distances in much the same way as small dsRNA fragments of RNA viral genomes induce systemic signaling for defense against viral pathogens (Jorgensen et al., "An RNA-based Information Superhighway in Plants," Science 279:1486-1487, Figure at 1486 (1998), attached herein as Exhibit 7). If RNA is the substrate for the SIN1-like proteins in plants, then a role in binding, cleavage, and subsequent unwinding of double-stranded RNA is implicated, and the RNase III and RNA helicase domains are clearly important in the functioning of the gene. Movement of a target RNA from the maternal sporophyte into the developing embryo in a *SIN1/SUS1/CAF* dependent manner could also explain the role of the sporophyte in embryogenesis. The function of SIN1 in transcriptional regulation is further supported by recent studies that implicate 21-25 nt dsRNA molecules in transcriptional repression of transgenic promoter sequences in plants (Mette et al., "Transcriptional Silencing and Promoter Methylation Triggered by Double Stranded RNA," EMBO J 19:5194-5201 at 5198-5199, bridging ¶ through 2nd ¶ (2000), attached herein as Exhibit 8); Mette et al., "Resistance of RNA Mediated TGS to HC-Pro, a Viral Suppressor of PTGS, Suggests Alternative Pathways for dsRNA Processing," Current Biology 11:1119-1123 at Abstract, and at 1122, last 2 ¶¶ (2001), attached herein as Exhibit 9).

9. As noted above, recent studies have implicated dsRNA in transcriptional control, which may involve gene silencing, in early developmental stages in plants. Given the molecular nature of the predicted protein product of SIN1

nucleic acid molecule, it is likely that the role of *SIN1* in early embryogenesis is to down regulate the activity of RNA targets required for early embryogenesis. Effects of the mutant alleles of *SIN1* are entirely consistent with such a function, and *sin1* point mutations, which I have mapped to the helicase C domain of the protein, establish the critical requirement of a functional RNA helicase domain for the SIN1 protein. It is highly likely that the conserved multi-domain structure of SIN1, taught in the present application, is found in early stage development-related genes throughout the plant world. Armed with the nucleotide sequence and structural/functional description of SIN1 as taught in the present application, it would be possible for a researcher to obtain a *SIN1*-like gene from any other plant.

**Expression of SIN1 Transgene Promotes Flowering**

10. To investigate if expression of the *SIN1* transgene could modulate development in a plant, the 6.2-kb full-length *SIN1* cDNA disclosed in my present patent application was placed under the constitutive Cauliflower Mosaic Virus 35S promoter (35S::*SIN1*) and the resulting construct (with kanamycin resistance marker for selection) was introduced into otherwise wild type *Arabidopsis thaliana* (Columbia ecotype) plants through *Agrobacterium*-mediated transformation using infiltration methods.

11. The resulting transformants were selected on plates containing kanamycin. The presence of the *SIN1* transgene was confirmed by PCR analysis, and PCR positive plants were self-crossed to make lines homozygous for the 35S::SIN1 transgene. Putative homozygous lines were subsequently confirmed by segregation analysis. Two of these homozygous transgenic lines clearly expressed the SIN1 protein, as determined by immunocytochemical staining of ovule and meristem sections (see lines 9 and 12 of Figure 3, showing transcript localization via *in situ* hybridization, attached hereto as Exhibit 10; Figure 4, showing protein localization via α-SIN1 antibody, attached hereto as Exhibit 11).

12. The *SIN1* transgene accelerated the time of change to the reproductive phase in two homozygous plant lines (see lines 9 and 12 of Table I, attached herein as Exhibit 12). Expression of *SIN1* transgene (6.2 kb cDNA under the control of 35S promoter) promoted early flowering, as assayed by the number of days it took the plants to bolt, as well as decreased the number of leaves produced per plant. All the transgenic plant lines produced significantly fewer leaves than non-transformed controls, indicating possible subtle effects on flowering time not

measurable by our techniques with this model plant. Unlike 35S::*LFY*, the lateral branches do not terminate into flowers, and single flowers do not develop in the axils of the rosette leaves, consistent with the fact that *SIN1*, unlike *LFY*, is not a floral meristem identity gene. Thus, *SIN1* is necessary for the vegetative to flowering transition, and expression from a transgene can independently promote flowering in *Arabidopsis*. This early flowering effect of SIN1 without additional effects on branching pattern is likely to be useful in crop engineering, because other early flowering genes, including *LFY* and *APETALA1*, that are in current use for developing early flowering crop engineering alter branching patterns and reduce the number of flower-bearing branches.

13. Fertility can be functionally defined as the onset of reproductive maturity. Flowering of plants is a measure of the onset of maturity, therefore, plants that flower sooner reach maturity sooner, which results in increased fertility. When plants were transformed with the 35S::*SIN1* expression construct (6.2 kb cDNA under the control of a 35S promoter), SIN1 transgenic plants were shown (see Table I, Exhibit 12) to have a shorter time to floral production than control plants. This demonstrates that the SIN1 nucleic acid molecule of the instant application can be used to modulate fertility in plants.

14. Fecundity relates to reproductive maturity in combination with the total number of seeds a mature plant can produce. Thus, decreasing the time to flowering with expression of the protein of the present invention is one factor of increased fecundity, as it increases time spent in the adult phase. The other factor, seed development, is also related to expression of the protein of the present invention, as this protein, when maternally expressed, appears to coordinate the expression of zygotic pattern formation in the embryo (Ray et al., "Maternal Effects of the *short integuments1* Mutation on Embryo Development," Dev Biol 180: 365-369 (1996)). Table I shows that *SIN1* transgenic plants that over-express SIN1 exhibit early flowering and decreased leave production. This result demonstrates that fecundity can be regulated by use of the *SIN1* transgene in plants to modulate fecundity.

15. All three weak loss-of-function alleles of *SIN1* (*sin1-1, sin1-2, caf-1)* have defects in floral patterning, although the extent of this defect is dependent on the background ecotype (Jacobsen et al, "Disruption of an RNA Helicase/RNAse III Gene in *Arabidopsis* Causes Unregulated Cell Division in Floral Meristems," Development 126:5231-5243 at 5235-5236 (1999)). The floral architecture of the 35S::*SIN1* lines was examined, but no defect was observed in any of the three lines

(see Table II, attached herein as Exhibit 13). The number of seed sets per flower was normal. Thus, with respect to flowering time, the over-expression of *SIN1* gives the opposite phenotype of the *sin1* loss-of-function mutations. These results together demonstrate that expression of SIN1 transgene can be used as an agronomic tool for accelerating flowering cycle without adversely affecting plant morphology, the number of flowers per plant, fertility or fecundity.

16. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 9/27/2002

Animesh Ray, Ph.D.